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Interaction of CD99 with Its Paralog CD99L2 Positively Regulates CD99L2 Trafficking to Cell Surfaces

Giri Nam,*† Young-Kwan Lee,*† Hye Yeong Lee,‡ Min Jung Ma,* Masatake Araki,*‡ Kimi Araki,*‡ Seungbok Lee,*§ Im-Soon Lee,*§ and Eun Young Choi*§

Mouse CD99 and its paralog CD99-like 2 (CD99L2) are surface proteins implicated in cellular adhesion and migration. Although their distributions overlap in a wide variety of cells, their physical/functional relationship is currently unknown. In this study, we show the interaction between the two molecules and its consequence for membrane trafficking of mouse (m)CD99L2. The interaction was analyzed by bimolecular fluorescence complementation, immunoprecipitation, and fluorescence resonance energy transfer assays. When coexpressed, mCD99 formed heterodimers with mCD99L2, as well as homodimers, and the heterodimers were localized more efficiently at the plasma membrane than were the homodimers. Their interaction was cytoplasmic domain–dependent and enhanced mCD99L2 trafficking to the plasma membrane regardless of whether it was transiently overexpressed or endogenously expressed. Surface levels of endogenous mCD99L2 were markedly low on thymocytes, splenic leukocytes, and CTL lines derived from CD99-deficient mice. Importantly, the surface levels of mCD99L2 on mCD99-deficient cells recovered significantly when wild-type mCD99 was exogenously introduced, but they remained low when a cytoplasmic domain mutant of mCD99 was introduced. Our results demonstrate a novel role for mCD99 in membrane trafficking of mCD99L2, providing useful insights into controlling transendothelial migration of leukocytes. The Journal of Immunology, 2013, 191: 5730–5742.

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Abbreviations used in this article: AU, arbitrary unit; B6, C57BL/6; BFA, brefeldin A; BPC, bimolecular fluorescence complementation; CD99L2, CD99-like 2; CD99L2S, CD99-like 2 (short form); FRET, fluorescence resonance energy transfer; h, human; HA, hemagglutinin; KO, knockout; m, mouse; MFI, mean fluorescence intensity; MHC I, MHC class I; PM-YFP plasma membrane–targeted yellow fluorescent protein; WT, wild-type; YFP, yellow fluorescent protein.

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hCD99 Ab, surface expression of MHC class I (MHC I) molecules was upregulated on human thymocytes but downregulated in the absence of hCD99 (11–13). Additionally, upon IFN-γ treatment of Jurkat cells, hCD99 was shown to associate with and increase the trafficking of MHC I to the cell membrane (14). The physical association of hCD99 proteins (both the long form and the alternatively spliced short isoform) with MHC molecules and tetraspanin CD81 is functionally linked to formation of an immunological synapse between B and T cells that induces T cell activation (15). hCD99 itself has been identified as a component of the lateral border recycling compartment of endothelial cells, moving to the site of transcellular diapedesis (16). The transmembrane region of hCD99, conserved among the CD99 and CD99-like molecules across species (7), was found to mediate the molecular association with MHC I and with Golgi-resident proteins, such as p230/golgin, during trafficking (14). These data regarding the involvement of hCD99 in molecular transport have suggested a possible role of mCD99 in molecular transport as well.

We and others have previously demonstrated that CD99 could form homodimers through extracellular domain–mediated interactions in mice (17) and humans (18). In this study, based on significant similarities between mCD99 and mCD99L2, we explored the possibility of interaction between the two molecules and subsequent implications. The results from this study revealed that mCD99 directly interacts with mCD99L2 to form a heterodimer via its cytoplasmic domain and subsequently enhances the surface expression of mCD99L2. Based on these findings, we propose a novel role for mCD99 as a conveyor protein carrying mCD99L2 cargo proteins via endomembrane trafficking.

**Materials and Methods**

**Mice**

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H60 congenic mice (B6.CH60) were gifts from Dr. Derry Roopenian (The Jackson Laboratory). The mCD99-deficient mouse strain B6.Cd99<sup>−/−</sup> (<p230/golgin 2Tf44meg>), in which the Cd99 gene locus is trapped by the insertion of pU-2T plasmid (19), was generated at the Institute of Resource Development and Analysis, Kumamoto University (Kumamoto, Japan) and maintained through mating with B6 mice. All of the mice were maintained under specific pathogen-free conditions at the Center for Animal Resource Development of Seoul National University College of Medicine (Seoul, Korea).

**Establishment of CTL lines and cell culture**

The establishment of CD8 CTL lines was performed as described previously (20). In brief, wild-type (WT) B6 or mCD99-deficient B6 mice were i.p. injected with 2 × 10<sup>7</sup> splenocytes from H60 congenic mice (B6.CH60). Then, the splenic CD8 T cells were harvested from the injected mice on day 7 after injection, cultured ex vivo with irradiated H60 congenic splenocyte feeder cells in the presence of recombinant IL-2 (50 U/ml; Sigma-Aldrich, St. Louis, MO), and maintained by periodic restimulation with irradiated feeder cells on a weekly basis. During the 7-d culture period of CTL line passage, CD8 T cells underwent activation and resting cycles. The activation (on day 5 after reactivation) and/or resting (on day 7 before reactivation) status of the CTL lines was monitored via cell counts and flow cytometric analysis of cell size and surface marker expression, such as that of CD44. Cells, including CD8 CTL lines, HEK293, and mouse L cells, were cultured in DMEM containing 5% FBS (HyClone Laboratories, Logan, UT) and antibiotics.

**DNA constructs**

Flag-<sup>+</sup>, hemagglutinin (HA)-, or Myc-tagged mCD99L2 genes were subcloned into pBluescript-VN and pBluescript-VC vectors (provided by Dr. Chang-Deng Hu, Purdue University, West Lafayette, IN) and then the DNA fragments containing epitope-tagged mCD99L2 genes fused with VN or VC sequences were subsequently subcloned into pCI-neo (Promega, Madison, WI) or pCDNA 3.1 (Invitrogen, Carlsbad, CA) expression vectors. VN vectors carrying CD99 tagged at the N terminus with Myc and constructs for domain mutants of CD99 have been described previously (17). The mCD99 and mCD99L2 genes were also cloned to generate fusion proteins with fluorescence proteins such as yellow (YFP), cerulean (CFP), or mCherry (Clontech, Mountain View, CA). Myc-tagged mCD99-YFP, CytMutCD99-YFP, and TmMutCD99-YFP genes were subcloned into the pcDNA3.1 expression vector for coimmunoprecipitation. YFP, mCD99-YFP, and CytMutCD99-YFP genes were subcloned into pcMSCV-puro (Clontech) for transduction. Plasma membrane–targeted YFP (PM-YFP) was a gift from Dr. Sanghee Chang (Seoul National University College of Medicine, Seoul, Korea).

**Transfection and transduction**

HEK293 cells, which were plated onto either six-well plates or poly-L-lysine–coated glass coverslips for flow cytometry or confocal microscopic analysis, respectively, were transfected with the respective DNA constructs using the calcium phosphate transfection method. For the introduction of the mCD99-YFP fusion gene into mCD99-deficient CTL lines, the cells were incubated with filtered retroviral supernatants that were harvested from Platinum-E cells (Cell Biolabs, San Diego, CA) transiently transduced with mCD99-YFP-pMSCV-puro, CytMutCD99-YFP-pMSCV-puro, or YFP-pMSCV-puro mock vector in culture medium supplemented with Polybrene (10 μg/ml; Sigma-Aldrich) and rhIL-2 (50 U/ml; Sigma-Aldrich). After 2 more days of culture with fresh medium, transduced CTL cells were restimulated for passage in the culture medium containing 1 μg/ml puromycin (Sigma-Aldrich) for selection. After three more rounds of CTL stimulation for passage, YFP<sup>+</sup> cells were sorted with a FACSAria (BD Biosciences, Franklin Lakes, NJ) and maintained with regular CTL passage on a weekly basis as described above.

**Coimmunoprecipitation and Western blotting**

Transfected HEK293 cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA). After preclumping with protein G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h, anti-Myc epitope (Santa Cruz Biotechnology) or anti-Flag epitope (Novus Biologicals, Littleton, CO) Ab was applied. Then the Ab-bound proteins were pulled down using protein G beads (Sigma-Aldrich) and eluted by boiling in sample buffer. The co-immunoprecipitants and lysates from transfected cells or mouse spleocytes were resolved on 10% SDS-PAGE gels and subjected to immunoblotting using anti-Myc epitope (Santa Cruz Biotechnology), anti-Flag epitope (Sigma-Aldrich), and anti-HA epitope (Applied Biological Materials, Richmond, BC, Canada) primary Abs and an HRP-conjugated goat anti-mouse secondary IgG Ab (KOMA Biotechnology, Seoul, Korea). Bound proteins were visualized using ECL reagent (Intron, Seoul, Korea).

**Flow cytometry**

For surface staining, cells were incubated with anti-CD99L2 (goat anti-CD99L2; R&D Systems, Minneapolis, MN) or monoclonal anti-Flag Ab (Sigma-Aldrich) in flow cytometry buffer (1× PBS containing 0.1% bovine calf serum [w/v] and 0.05% sodium azide) at 4°C for 1 h, washed, and, with stained with PE-conjugated secondary Abs at 4°C for 30 min. PE-conjugated anti-mCD99 Ab (E2; rat anti-mCD99) was used for staining of mCD99. For staining both surface and intracellular mCD99, cells were fixed with 4% paraformaldehyde in 1× PBS, permeabilized with 0.5% saponin in 1× PBS, and, stained with Abs. Stained cells were washed and analyzed following dead cell exclusion by DAPI staining using an LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). Additional Abs used for flow cytometry were alkalinephosphatase-labeled anti-rat anti-Cy5 (53-6.7), alkalinephosphatase–conjugated rat anti-mouse CD3 (145-2C11), PE–conjugated mouse anti–mouse H-2K<sup>B</sup> (AF6-88.5; all from BD Biosciences), alkalinephosphatase–conjugated rat anti-CD4 (GK1.5), PE–conjugated rat anti-mouse B220 (RA3-6B2; eBioscience, San Diego, CA), and PE–conjugated donkey anti-goat IgG (R&D Systems). For inhibition of molecular transport from the Golgi to the plasma membrane or recycling at the plasma membrane, transfected HEK293 cells or transduced CD8 CTL cells were treated with 3 μg/ml brefeldin A (BFA; eBioscience) or 40 μM Dynasore (N-3,4-dihydroxybenzylidene)-3-hydroxy-2-naphthylhydroxazide; Santa Cruz Biotechnology), respectively, for 4 h prior to flow cytometry analysis. Cells treated with 0.1% DMSO (Sigma-Aldrich) were also included as a control.

**Confocal microscopy assay**

For assays that were analyzed by confocal microscopy, transfected HEK293 cells were washed with 1× PBS, fixed with 4% paraformaldehyde for 5 min, and, after washing, incubated with BODIPY TR ceramide (N-(4-
Results

Formation of heterodimeric biomolecular fluorescence complementation complexes composed of mCD99 and mCD99L2

To investigate the potential interaction of mCD99 and mCD99L2, we first performed biomolecular fluorescence complementation (BiFC) analysis, which enables direct visualization of protein interactions as well as subcellular localization of protein complexes in living cells via the fluorescence caused by the association of two proteins fused to nonfluorescent fragments of a fluorescent protein (21). mCD99 and two isoforms of mCD99L2 (mCD99L2S and mCD99L2L) were tagged with Myc, Flag, or HA epitopes, not only because Abs that can differentiate the CD99L2 isoforms are not available, but also because the anti-CD99 mAb raised in our laboratory (EJ2) was not of sufficient high affinity for use in certain analyses, such as immunoprecipitation. Next, the tagged genes were fused to either a VN (N-fragment of the yellow fluorescent protein Venus) or a VC (C-fragment of Venus) fragment for BiFC analysis (Fig. 1A). The sizes and expression levels of the VN- and VC-fused forms of CD99L2S, CD99L2L, and CD99 were confirmed by immunoblotting (data not shown).

We next transfected HEK293 cells with mCD99-VN and mCD99L2-VC constructs in pairs and analyzed the transfected cells by flow cytometry and confocal microscopy to detect any physical interactions. Additionally, cells were cotransfected with mCD99 plus hCD4 or with Jun plus Fos (in VN and VC combinations) as negative or positive control, respectively. When the cells were subjected to flow cytometric analysis 48 h after transfection, the results revealed that the cells transfected with mCD99-VN and mCD99L2S-VC, as well as with mCD99L2S and mCD99L2L-VC, eminated Venus fluorescence, implying that mCD99 and mCD99L2 molecules formed mCD99/mCD99L2 heterodimeric complexes.

Statistical analysis

Statistical analysis was performed using two-tailed t tests by GraphPad Prism software (version 5; GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.
The fluorescence intensities were comparable with those resulting from homodimeric interactions between mCD99-VN and mCD99-VC (Fig. 1B). The mCD99–mCD99L2 interaction was confirmed by coimmunoprecipitation assays, in which lysates from the cells cotransfected with Myc-tagged mCD99-VN plus Flag-tagged mCD99L2S-VC (or Flag-tagged mCD99L2L-VC) were precipitated using an anti-Myc Ab. Western blotting with an anti-Flag Ab yielded specific bands corresponding to mCD99L2 in both the short and long forms (Fig. 1C). Lysate from cells cotransfected with mCD99-HA-VC and Myc-mCD99-VN was used as a positive control.

By using confocal microscopic analysis that provides qualitative in-focus images of BiFC complexes, we detected the formation of mCD99/mCD99L2S and mCD99/mCD99L2L heterodimeric BiFC complexes. The heterodimeric complexes were localized to the plasma membrane and to the Golgi complex in transfected cells, as determined by coimmmunostaining Venus fluorescence with membrane dye DiI (Fig. 1D) and Golgi dye BODIPY (Supplemental Fig. 1), respectively. No notable differences in heterodimer formation with mCD99 were observed between the two mCD99L2 isoforms. The subcellular localization of the heterodimeric complexes appeared to be more peripheral than that of mCD99/mCD99 homodimeric complexes; the latter showed a more concentrated pattern at the endoplasmic reticulum and Golgi regions, suggesting that mCD99–mCD99L2 heterodimers move farther along the endomembrane system than did CD99 homodimers.

We next tested whether mCD99L2 proteins themselves could form homodimers (mCD99L2S-mCD99L2S, mCD99L2L-mCD99L2L, or mCD99L2S-mCD99L2L), as does mCD99 (17). On the contrary to mCD99, none of the three CD99L2 protein combinations formed homodimers according to BiFC-based fluorescence detected via flow cytometry (Fig. 1B) or confocal microscopic (Fig. 1D) analysis. This suggests that, unlike mCD99, mCD99L2 proteins do not form homodimeric BiFC complexes, which was subsequently confirmed by coimmunoprecipitation analysis (Fig. 1C).

Taken together, the results of our BiFC analyses showed that mCD99 and mCD99L2 physically interacted with each other to form heterodimeric complexes with a subcellular distribution pattern distinct from that of mCD99–mCD99 homodimers. When we used additional pairs of VN- and VC-fused constructs, in which the CD99 and CD99L2 gene constructs used were the reciprocals of those used in the above experiments (Fig. 1B–D), we obtained similar results by BiFC analysis (data not shown).

Specific interaction of mCD99 and mCD99L2 in an acceptor photobleaching FRET assay

Although the BiFC assay has been reported to be so sensitive as to detect not only transient but also very weak interactions (22), we tried to confirm the interaction between mCD99 and mCD99L2 using another method, the FRET assay, which can detect protein–protein interactions within a 1–10 nm range (23–25). We cotransfected HEK293 cells with constructs to express mCD99 and mCD99L2 proteins tagged with YFP and CFP at the C terminus, respectively (Fig. 2A). Additionally, cells were transfected to coexpress other combinations of YFP- or CFP-fused mCD99 and mCD99L2 proteins to check for homodimerization between mCD99 proteins or between mCD99L2 proteins; coexpressed mCD99 and hCD4 fusion proteins were used as a no interaction (negative) control. We examined whether the emission of CFP donor signals became more intense after photobleaching of the YFP acceptor within the intracellular and plasma membrane regions of the cotransfected cells. The signal quench of the YFP acceptor by photobleaching enables the CFP donor signal to be directly detected without being diverted (i.e., absorbed) for YFP acceptor activation (24). Comparison of the cell images taken before and after the localized photobleaching of the YFP acceptor, which were then processed into spectrally unmixed images, revealed that in the cells cotransfected with mCD99-YFP and mCD99L2-CFP DNA constructs, the intensity of the mCD99L2-CFP donor signal increased after photobleaching, but only within the photobleached regions and not in nonphotobleached regions (Fig. 2B). The FRET efficiency values observed in the bleached areas were significant (mean, 21.5 ± 0.7 arbitrary units).

![FIGURE 2](http://www.jimmunol.org/content/jimmunol/185/9/5733.full.pdf)
[AU]; \( n = 26 \)), indicating the occurrence of FRET in the cells and thereby confirming the interaction of mCD99 and mCD99L2 (Fig. 2C). FRET was also observed in the photobleached regions of cells coexpressing YFP- and CFP-fused mCD99 proteins, with significantly high efficiency (mean, \( 32.90 \pm 2.0 \) AU; \( n = 14 \)), but not in those coexpressing hCD4 and mCD99 fusion proteins or in those coexpressing CFP- and YFP-tagged mCD99L2, verifying the lack of interaction between mCD99L2 proteins themselves. Altogether, the results from the FRET assays proved the existence of molecular interactions between mCD99 and mCD99L in the cells where they were coexpressed. Furthermore, the molecular specificity for dimerization was validated to occur between mCD99L2 and mCD99 molecules as well as between mCD99 molecules, consistent with the results of the BiFC analysis.

Coexistence of mCD99-mCD99 and mCD99-mCD99L2 dimers within a cell

Because mCD99 interacts not only with itself but also with mCD99L2 to form dimers, we tested which partner it preferred for dimer formation. Using BiFC and FRET analyses, we detected a slight difference in the pattern of subcellular localization between homodimeric and heterodimeric complexes; heterodimers appeared more frequently at the plasma membrane than did homodimers (Figs. 1D, 2B). To directly compare the subcellular localizations of the two different dimers, we performed multicolor BiFC analysis, because two different fluorescence colors (YFP and CFP) can be simultaneously expressed in a cell through interaction of VN- and CC-tagged proteins and of CN- and CC-tagged proteins, respectively (26). HEK293 cells were cotransfected with three DNA constructs for the expression of mCD99-VN, mCD99-CC, and mCD99L2-CN proteins (Fig. 3A). Our speculation was that if mCD99 preferred one partner over the other, only a single fluorescence signal, YFP or CFP, would be expressed in the transfected cells. As single-color controls, cells were cotransfected with two of the DNA constructs to express VN and CC fusion proteins (for emitting YFP only) or CN and CC fusion proteins (for emitting CFP only). Upon examining the three-construct transfected cells under confocal microscopy, we observed that, although most cells emanated both fluorescent colors, the signal intensities of YFP and CFP varied, indicating the formation of different levels of the two dimers depending on the status of individual cells. To quantify each signal, we converted the absolute values of YFP and CFP intensities produced by a single cell into a relative YFP value (YFP/\( \text{YFP + CFP} \)) and were thus able to evaluate \( >90 \) different cells according to their relative YFP values. When we plotted cell numbers versus relative YFP values, we found that whereas the distribution of single-color control cells skewed toward 0.1–0.2 (i.e., heterodimers producing CFP) or 1 (i.e., homodimers producing YFP), that of the three-construct transfected cells varied widely from 0.1 to 0.9 of the relative YFP values, indicating expression of both colors (i.e., the coexistence of heterodimers and homodimers) at varying ratios in cells coexpressing mCD99-VN, mCD99-CC, and mCD99L2-CN proteins (Fig. 3B, Suplementary Fig. 2A).

Next, we compared images of cells emitting equal levels of YFP and CFP signals to examine the subcellular localizations of the two types of dimers. Individual nonoverlapping cell images showed that mCD99-mCD99L2 heterodimers broadly distributed from the perinuclear region to the plasma membrane, whereas mCD99 homodimers were more restricted near perinuclear vesicles (Fig. 3C, Supplementary Fig. 2B). Thus, major heterodimeric CFP peaks with \( >500 \) intensity AU were measured at the perinuclear regions (5.9, 6.1, 6.3, 16.6, 17.1, 17.2, and 17.6 \( \mu \)m from one end of a cell along an arbitrary line crossing the nucleus) and at more peripheral regions (2.1, 2.9, and 19.3 \( \mu \)m from the end of a cell). Additionally, major homodimeric YFP peaks were detected at 5.9, 6.1, 6.2, 16.9, 17.1, 17.2, and 17.3 \( \mu \)m from the end of a cell (in the perinuclear region) with strong intensities (\( >1000 \) AU). These data provide clear evidence for different localization patterns of the two types of dimers: more focused perinuclear localization of homodimers versus more diffuse, peripheral, but distinct perinuclear localization of heterodimers (Fig. 3D, Supplementary Fig. 2C).

Altogether, the results from the multiple BiFC and subsequent analyses demonstrated that mCD99-mCD99L2 heterodimers and mCD99-mCD99 homodimers coexist within the same cell with differing localization patterns.
The ability of mCD99 to form two different types of dimers within an individual cell led us to question which domain of mCD99 was responsible for interaction with mCD99L2 because its extracellular domain has been found to mediate homodimer formation in domain-mutant analysis using BiFC assays (17). To answer this question, we performed BiFC analyses with VN- and VC-fused combinations of mCD99L2 and mCD99 domain mutants, in which the extracellular, transmembrane, or cytoplasmic domain (ExtMutCD99, TmMutCD99, or CytMutCD99, respectively) was replaced with the corresponding domain of hCD4 (17). Flow cytometric analysis of HEK293 cells cotransfected with mutant mCD99 and mCD99L2 constructs revealed a marked reduction in BiFC-based fluorescence in cells expressing CytMutCD99 together with mCD99L2. However, other BiFC combinations of mCD99 mutants yielded strong fluorescent signals (Fig. 4A).

Next, to quantify the difference in the degrees of interaction with mCD99L2 between different types of mCD99, we cotransfected HEK293 cells with three DNA constructs for the expression of mCherry fluorescence protein to mark transfected cells in addition to VN- and VC-fused mCD99L2 and mCD99 expression, and counted cells emitting Venus fluorescence among the mCherry+ transfected cells in five different fields under a fluorescence microscope. When numbers of the Venus-expressing cells per 100 mCherry+ cells in each field were processed to a plot, the formation of BiFC complexes in cells expressing CytMutCD99 together with mCD99L2 was determined. However, other BiFC combinations of mCD99 mutants yielded strong fluorescent signals (Fig. 4A).

To verify the results obtained from confocal microscopic analysis, we compared the level of mCD99L2 expressed on the cell surface under the two conditions (the presence and absence of mCD99 coexpression) by flow cytometry after staining the transfected cells with anti-mCD99L2 Ab. Results from the flow cytometric analysis, shown as overlaid single histograms (Fig. 5C), demonstrated a shift to the right of the histograms corresponding to mCD99L2 expression (Fig. 5C). This indicated enhanced localization of mCD99L2 at the surface of cells coexpressing mCD99 and mCD99L2 was significantly reduced by the absence of original cytoplasmic domain of mCD99.

In coimmunoprecipitation assays using lysates from cells transfected to coexpress Flag-tagged mCD99L2-YFP and each type of the Myc-tagged mCD99-YFP proteins, immunoblots of precipitants of anti-Flag Ab with anti-Myc Ab showed that intensities of the bands corresponding to CytMutCD99 were relatively low, on average 0.6-fold of those corresponding to WT or TmMut mCD99-VC. However, the complex occurred in <20% of the cells transfected to express CytMutCD99-VC and mCD99L2-VN pairs (Fig. 4B), a lower frequency than those obtained from other combinations but still significant compared with the negative control where hCD4 was expressed as VC partner to mCD99L2-VN. Therefore, these results suggest that the efficiency or affinity of the interaction between mCD99 and mCD99L2 was significantly reduced by the absence of original cytoplasmic domain of mCD99.

Additionally, confocal microscopy of the cells expressing BiFC-based Venus fluorescence revealed that, despite the infrequent formation, BiFC complexes once formed by mCD99L2 with the cytoplasmic domain mutant of mCD99 were localized mostly at intracellular vesicles (Fig. 4D), whereas those with the other types of mCD99 were localized to the plasma membrane as well as intracellular vesicles. This observation implied the significance of the cytoplasmic domain of mCD99 in the localization of the complex formed between mCD99 and mCD99L2.

Enhanced localization of mCD99L2 at the plasma membrane via coexpressed mCD99

The functional significance of the interaction of mCD99 and mCD99L2 was next addressed. Based on previous reports on the role of hCD99 in controlling MHC I trafficking (12, 14, 15) and our results showing the more peripheral localization of the heterodimer (Fig. 3), we investigated a role for mCD99 in trafficking of mCD99L2. To explore the influence of the interaction with mCD99 on mCD99L2 trafficking, we compared the level of mCD99L2 localized at the plasma membrane between cells expressing mCD99L2 alone and those coexpressing mCD99L2 and mCD99. HEK293 cells were transiently transfected to express an mCD99L2-mCherry fusion protein in combination with a native unfused CFP control protein or with an mCD99-CFP fusion protein, respectively, and analyzed for localization of mCD99L2 at the cell membrane via confocal microscopy. To visualize the plasma membrane region of transfected cells, a chimeric construct of the 10 N-terminal amino acids of Lyn with YFP (PM-YFP), which is targeted to the inner monolayer of the plasma membrane (27, 28), was included. Then, colocalization of mCherry (mCD99L2) and YFP (PM) was considered indicative of mCD99L2 localization at the plasma membrane. Confocal images taken 48 h after transfection demonstrated that expression of mCD99L2 at the plasma membrane, as denoted by colocalization with PM-YFP, was not significantly high when coexpressed with native CFP, but it was substantially enhanced when coexpressed with mCD99-CFP (Fig. 5A). According to a quantitative analysis (29), the colocalization of mCD99L2-mCherry with PM-YFP increased 1.5-fold, on average, by the presence of mCD99 (mean colocalization r value, 0.67) compared with that in the absence of mCD99 coexpression (mean r value, 0.44; Fig. 5B). These results indicated a positive impact of mCD99 coexpression on the localization of mCD99L2 to the plasma membrane.

To verify the results obtained from confocal microscopic analysis, we compared the level of mCD99L2 expressed on the cell surface under the two conditions (the presence and absence of mCD99 coexpression) by flow cytometry after staining the transfected cells with anti-mCD99L2 Ab. Results from the flow cytometric analysis, shown as overlaid single histograms (Fig. 5C), demonstrated a shift to the right of the histograms corresponding to mCD99L2 expression (Fig. 5C). This indicated enhanced localization of mCD99L2 at the surface of cells coexpressing mCD99 and mCD99L2 (average relative mean fluorescence intensity [MFI], 3.0), with a 1.8-fold increase in MFI compared with cells expressing mCD99L2 alone (average relative MFI, 1.7). A similar increase in mCD99L2 surface expression (average relative MFI, 2.9 versus 1.9; 1.5-fold) was obtained when the cells were stained with anti-Flag Ab, which was used to detect the Flag epitope-tagged mCD99L2 (Fig. 5D). This result ruled out the possibility of false positives due to cross-reactivity of the anti-CD99L2 Ab to the mCD99 protein expressed on the cotransfected cells. Taken together, the results from the confocal microscopic and flow cytometric analyses using cotransfected HEK293 cells demonstrated that localization of mCD99L2 to the plasma membrane was enhanced by coexpression of mCD99, suggesting a positive role for mCD99 in molecular trafficking of mCD99L2 to the plasma membrane.

Next, to determine the implication of mCD99 in molecular trafficking of interacting mCD99L2 from the Golgi to the plasma membrane, HEK293 cells were transfected to express mCD99 and mCD99L2 together or each molecule alone with BFA (a blocker of Golgi to membrane trafficking), Dynasore (a blocker of capping), and DMSO control solution for 4 h and then stained with anti-mCD99 or anti-mCD99L2 Ab for flow cytometric analysis. Results from flow cytometry analysis showed that, compared with levels expressed on cells treated with DMSO or Dynasore, the surface level of mCD99 itself was significantly reduced by BFA treatment whether it was expressed alone or together with mCD99L2 (Fig. 5E). In the case of mCD99L2, BFA treatment did not influence surface levels of mCD99L2.
when the molecule was expressed alone, but it did reduce the levels when it was coexpressed with mCD99. The MFI values of mCD99L2 on the BFA-treated cells that coexpressed mCD99 and mCD99L2 were low compared with those of cells treated with DMSO or Dynasore (Fig. 5F). Taken together, these data suggest that surface level of mCD99 is dependent on the trafficking activity from the Golgi to the plasma membrane on its own, and surface expression of mCD99L2 is enhanced via the activity of mCD99 upon interaction.

**Increased transport of endogenously expressed mCD99L2 to the plasma membrane via interaction with mCD99**

The positive effect of coexpressed mCD99 on the peripheral localization of mCD99L2 (Fig. 5) was determined using transiently...
transfected HEK293 cells. Thus, we next sought to confirm those results in cells that endogenously express mCD99L2. The mouse L cell line, which expresses mCD99L2 but lacks mCD99 expression (Fig. 6A), was transfected with mCD99-YFP and analyzed for endogenous mCD99L2 expression on the cell surface by flow cytometry after staining with anti-mCD99L2 Ab. Data from the flow cytometric analysis demonstrated that compared with cells mock-transfected with native YFP and untransfected YFP cells, the YFP+ mCD99-transfected cells expressed more mCD99L2 on their surface; mCD99L2 surface expression was 1.3-fold greater, on average, in the presence of mCD99-YFP (Fig. 6B). Even though the increase was not dramatic, possibly due to a limit on endogenously expressed mCD99L2 protein, the phenomenon was consistently and significantly reproducible.

To examine whether this effect of mCD99 coexpression was due to the interaction of mCD99 with mCD99L2, we also transfected L cells to express CytMutCD99-YFP, which has a limited ability to interact with mCD99L2 (Fig. 4), followed by staining with anti-mCD99 Ab or anti-mCD99L2 Ab for flow cytometric analysis. To confirm the expression of CytMutCD99 on the surface of the transfected (YFP+) cells, we used flow cytometry analysis after staining with anti-mCD99 Ab and revealed that trafficking of CytMutCD99 itself to the plasma membrane was inefficient (Fig. 6A); the level of CytMutCD99 protein on the surface of YFP+ cells was low when the mutant proteins were present in a low to intermediate concentration (YFPint), and was high, comparable to those of the WT mCD99, when the mutant protein was at a high concentration (YFPhi). This surface expression pattern for CytMutCD99 was different from that of WT mCD99, which showed a proportional increase relative to YFP intensity, suggesting a differential molecular trafficking pattern of the mutant protein, in addition to inefficient interaction with mCD99L2.
Additionally, flow cytometric analysis after staining with anti-mCD99L2 Ab showed that CytMutCD99-expressing cells displayed mCD99L2 on their surface at lower levels in the YFPhi population as well as in the YFPint population than WT mCD99-expressing cells (Fig. 6B).

Next, we stained cells with anti-mCD99 Ab after membrane permeabilization to detect proteins present inside and outside of the cell to determine whether CytMutCD99 and WT mCD99 proteins were produced at similar levels in the transfected cells. After gating on YFP+ cells, flow cytometric analysis confirmed that the transfected cells produced CytMutCD99 and mCD99 proteins in similar amounts, indicating that the cytoplasmic domain mutant mCD99 indeed could not reach the cell surface as efficiently as did the WT mCD99 protein (Fig. 6C). These data were in agreement with the aforementioned results from confocal microscopic analysis (Fig. 4D), suggesting that the low level of mCD99L2 expressed on the surface of CytMutCD99-transfected cells was due to the inefficient trafficking of CytMutCD99 itself and/or insufficient interaction between the CytMutCD99 and mCD99L2.

Taken together, these results demonstrated that forced expression of mCD99 enhanced the trafficking to the plasma membrane of endogenous mCD99L2 and this positive effect was dependent on the cytoplasmic domain of mCD99.

**Reduced surface expression of mCD99L2 on cells from CD99-deficient mice**

Because the above results demonstrated the role of mCD99 in terms of gain-of-function analysis, we next questioned whether this function of mCD99 would be revealed via loss-of-function experiments. To address this question, we obtained mice in which the Cad99 gene was trapped by a random pUT-21 plasmid insertion and was consequently not translated (19). Using these mice, we performed flow cytometry to detect the surface expression of mCD99L2. Cells from the thymi and the spleens of CD99-deficient mice displayed no abnormal phenotypic features under normal conditions (without antigenic or pathologic stimulation; data not shown). However, flow cytometric analysis revealed that total thymocytes and splenocytes from the CD99-deficient mice displayed reduced surface levels of mCD99L2 (Fig. 7A, 7B). The levels of mCD99L2 on CD99-deficient thymocytes and splenocytes were 0.3- and 0.5-fold reduced, respectively, compared with those on WT cells. When CD4 and CD8 single-positive thymocytes (minor populations in the thymocytes) and CD3+ T cells or B220+ B cells (two major populations in the splenocytes) were analyzed for the mCD99L2 surface levels via flow cytometry, we observed the same phenotype that mCD99L2 levels were comparatively lower on the CD99-deficient cells (Supplemental Fig. 3). The reduction in mCD99L2 surface expression was in contrast to the consistent surface levels of MHC I (H-2Kb), a target molecule related to the transport activity of hCD99 (12, 14, 15), regardless of the presence of mCD99 expression (Fig. 7A, 7B). When flow cytometric analysis was performed after staining the membrane-permeabilized cells with anti-mCD99L2 Ab, overall mCD99L2 protein levels were found to be comparable between mCD99-deficient and WT cells (Fig. 7C, 7D), implying that the difference in surface levels of mCD99L2 between the two types of cells was not due to differences in production of the mCD99L2 protein. Based on immunoblot and RT-PCR analyses using the lysates of WT or CD99-deficient splenocytes.
to detect mCD99L2 proteins and transcripts, respectively, the amounts of those produced by CD99-deficient cells were comparable to those produced by WT cells (Supplemental Fig. 4), supporting the results obtained from flow cytometric analysis after intracellular staining.

The levels of both mCD99 and mCD99L2 were not sufficiently high on the naive ex vivo cells and, therefore, the difference in surface mCD99L2 expression between the WT and mCD99-deficient cells was not dramatic (Fig. 7A, 7B). Because expression of mCD99 is upregulated on activated T cells (17), we pos-

Collectively, these results demonstrated the negative influence of mCD99 deficiency on surface expression of mCD99L2 specifically and confirmed the involvement of mCD99 in the molecular transport of mCD99L2 to the plasma membrane.

Increased mCD99L2 expression on mCD99-deficient cells after mCD99 gene transduction

Finally, we tested whether the level of mCD99L2 expressed on the surface of mCD99-deficient cells could be reestablished when mCD99 expression was reinstated. To this end, mCD9-deficient CTL cells were retrovirally transduced to express mCD99-YFP and then stained with anti-mCD99 and anti-mCD99L2 Abs to check their expression levels on the surface of YFP+ cells by flow cytometry. The flow cytometry data showed that surface mCD99 expression increased 1.9-fold, on average, after the transduction of mCD99-YFP compared with nontransduced or mock-transduced mCD99-deficient cells, even though the increased expression did not reach the level expressed by WT cells (Fig. 8A, 8B). The surface level of endogenous mCD99L2 on mCD99-deficient cells increased 1.3-fold, on average, after the mCD99-YFP transduction (Fig. 8A, 8B), but it was still lower than on WT CTLs. Such a significant increase in the surface expression of endogenous mCD99L2 on the mCD99-deficient cells was not observed after the transduction with YFP-fused cytoplasmic domain mutant of mCD99 (Fig. 8A, 8B), which was considered to be due to the low capability of the mutant to interact with mCD99L2 and its inefficient trafficking to the plasma membrane. In support of this notion, when we treated CTLs transduced to express YFP-fused WT or the cytoplasmic domain mutant type of mCD99 with BFA, we found that the BFA treatment reduced the levels of endogenous mCD99L2 on the cells transduced with mCD99-YFP, as well as surface levels of the exogenous mCD99L2-YFP (Fig. 8C, 8D). However, such a reduction in mCD99L2 surface levels was not observed when the cells reconstituted with CytMutCD99-YFP were treated with BFA.

Altogether, the results, which demonstrated fluctuation in the surface level of mCD99L2 according to expression of WT of mCD99, confirmed the supportive role of coexpression of and the interaction with mCD99 in the membrane localization of endogenous mCD99L2.

Discussion

In this study, we demonstrated that mCD99 and mCD99L2 interact to form heterodimers and that this interaction enhances the trafficking of mCD99L2 to the plasma membrane. This is the first demonstration of involvement of mCD99 in intracellular mCD99L2 transport.

As initially postulated by the fact that hCD99 participates in the molecular transport of several surface molecules via physical association, we confirmed physical association of mCD99 with mCD99L2 using BiFC, FRET, and immunoprecipitation assays. We also observed that mCD99L2 transport to the plasma membranes is increased by interaction with mCD99 regardless of
whether the former is expressed exogenously or endogenously. The significance of mCD99 involvement in the molecular transport of mCD99L2 was verified by modulation of mCD99L2 surface levels depending on the presence or absence of mCD99. However, unlike hCD99, the positive effect of mCD99 coexpression was not extended to membrane trafficking of MHC I, the most intensely investigated target protein in terms of the trafficking-related function of hCD99 (12, 14, 15, 30). Rather, the surface levels of the mouse counterpart (H-2Kb) were steadily maintained on thymocytes, splenocytes, and CTLs regardless of mCD99 expression. Moreover, the transmembrane domain of CD99, which mediates the association of hCD99 with HLA and Golgi-resident p230/golgin-245 proteins (14, 31–33), was dispensable for the interaction of mCD99 with mCD99L2. These discordant features of hCD99 and

**FIGURE 8.** Restoration of mCD99 and mCD99L2 on the surface of CD99-deficient CTL lines by overexpression of mCD99. (A) Flow cytometric analysis of the restoration of mCD99 and mCD99L2 expression on CD99-deficient CTL cells by retrovirus-mediated mCD99-YFP expression. CD99-deficient (CD99KO) CTL cells that were transduced with retrovirus expressing Mock-YFP (CD99KO + Mock-YFP), mCD99-YFP (CD99KO + mCD99-YFP), or CytMutCD99-YFP (CD99cyt-YFP) were selected in culture medium containing puromycin and subsequently sorted for YFP+ cells using a FACSaria. Sorted cells were stained with PE-conjugated anti-CD99 or anti-CD99L2 Ab plus donkey anti-goat IgG secondary Ab and analyzed by flow cytometry using an LSR II flow cytometer. (B) Comparison of relative mCD99 and mCD99L2 MFI values. Relative MFI values of mCD99 and mCD99L2 on WT or on mCD99-YFP-transduced (+CD99) or CytMutCD99-YFP-transduced (+CD99cyt) CD99KO cells were obtained in comparison with MFI values on CD99KO or on CD99KO plus Mock-YFP (+Mock) cells, respectively, and were plotted. Data (A, B) represent the results obtained from three independent experiments. ***p < 0.001. (C and D) Flow cytometric analysis was performed to detect surface levels of mCD99 and mCD99L2 expressed in CD99-deficient CTL cells transduced to express Mock-YFP, mCD99-YFP, or CytMutCD99-YFP after treatment with DMSO or BFA. DMSO (0.1%) or BFA (3 μg/ml) was added to transduced CTL cells on regular stimulation for cell passage for 4 h. Then, the cells were harvested, washed, and stained with anti-CD99 Ab (C) or anti-CD99L2 Ab (D), in addition to anti-CD8 Ab. Cells were analyzed via flow cytometry after gating YFP+ and CD8+ cells, and the MFI values for mCD99 (C) or mCD99L2 (D) were plotted. Data shown (C, D) represent three independent experiments. *p < 0.05.
mCD99 suggest that CD99 may have diverged in its function with regard to MHC I trafficking. It is also possible that the function of mCD99 in regulating the trafficking of CD99L2 may be unique to mouse cells. Thus, further investigation on the relationship between hCD99 and hCD99L2 and detailed characterization of mCD99 at the molecular level are necessary to clarify these discrepancies.

In our study, the intact cytoplasmic domain of mCD99 was required not only for sufficient interaction with mCD99L2, but also for efficient membrane trafficking of both mCD99 itself and mCD99L2. Because the cytoplasmic domain of mCD99 is too short to contain any signaling motif, recruitment of a signaling molecule may be necessary for further events such as facilitating cytoskeletal reorganization for the transport of cargo proteins. In contrast to mCD99, mCD99L2 proteins (both the short and long forms) have a longer cytoplasmic domain that contains a proline-rich (EPPP-PEPPPR) sequence (7). Because this motif is recognized by SH3 domains, mCD99L2 itself may play a certain role in the signaling pathways that control cytoskeletal reorganization. Therefore, the cytoplasmic domain–mediated interaction with mCD99 may stabilize or enhance signaling through the cytoplasmic motif of mCD99L2, consequently upregulating the membrane trafficking of mCD99L2. Currently, we are generating various mutants with serial deletions or amino acid substitutions at the cytoplasmic regions of mCD99 and mCD99L2 to pinpoint amino acids critical for their interaction and membrane trafficking of both molecules.

While screening various types of peripheral blood leukocytes from normal B6 mice via flow cytometry, we found that most of the cells were dual-positive cells that express both CD99 and CD99L2 (data not shown). However, single-positive cells were also identified, such as mCD99+ mCD99L2+ bone marrow–derived neutrophils (8) and mCD99+ mCD99L2+ L cells (Fig. 6), suggesting the presence of mCD99 (homodimer and heterodimer) and mCD99L2 (monomer and heterodimer) in various forms at the plasma membrane. Molecular existence and trafficking to plasma membrane in homo- and heterodimeric forms have been reported for some chemokines. Dynamic interplay between these complexes at the cell membrane is considered as adaptation to changes in milieu. CXCR1 and CXCR2, for instance, can form homo- and heterodimers with similar efficiency (34, 35). Homo- and heterodimers of CXCR1 and CXCR2 coexist at the cell surface in the absence of ligand binding. However, ligand CXCL8 disturbs the heterodimers and stabilizes both homodimeric forms. Then, the CXCL8-mediated activation triggers CXCR2 internalization more rapidly owing to its high affinity for CXCR2, leading to rapid reduction of CXCR2 and, relatively, persistence of CXCR1 at the cell membrane (36). CCR5 is mainly maintained within the intracellular compartment of Jurkat cells when it forms homodimers, but it is exported to the plasma membrane by its association with CD4 (37). Membrane trafficking of CCR5 homodimers and CCR5–CXCR4 heterodimers relies on different Rab GTPases, depending on the presence or absence of CD4 (38). Likewise, a shift in molecular forms of mCD99 and mCD99L2 may occur dynamically according to the functional needs or depending on availability of other partner molecules.

An additional role of mCD99, implication in cell death, has been reported; mCD99+ cells were killed through the recognition of mCD99 by PILRα-expressing NK cells (39), and the ligation of mCD99 to the polymeric fusion protein PILR-FC induced apoptosis in immature thymocytes (40), as is implicated in apoptosis of thymocytes by hCD99 (41), Ewing sarcoma cells (42, 43), and lymphoblast leukemia cells (44). Given the physiologic heterodimeric and homodimeric forms of mCD99, therefore, the identity of CD99 family dimers dominant on the cell surface under normal physiological conditions, during inflammation-induced diapedesis, or during apoptosis would be necessary to be defined to understand the biological relevance of CD99 oligomerization and, ultimately, the role of CD99 protein, and this is currently under investigation.

To date, conflicting reports exist on the role of mCD99L2 in homophilic cell adhesion. Transient mCD99L2 gene transfection has been reported to induce homotypic adhesion of mouse L cells (9), but not that of Chinese hamster ovary cells (8). mCD99L2-transfected HEK293 cells also failed to show significant aggregation in our experiments (data not shown). This discrepant role of mCD99L2 in homophilic cell aggregation might be attributable to the use of different cell lines by the different studies, including concerns of animal origin: mouse versus hamster versus human. With the unique roles of mCD99L2 still unidentified, evaluation of the influence of mCD99 coexpression on the function of mCD99L2 was limited. The overlap in known functions of mCD99 and mCD99L2 (which are both implicated in cell adhesion and migration) hampered the design of an experiment that could discriminate between functional enhancement of mCD99L2 by mCD99 coexpression and an additive functional effect. We elucidated the positive influence of mCD99 coexpression on membrane trafficking of mCD99L2. This finding, combined with the fact that mCD99 and mCD99L2 function at the same site and stage during leukocyte diapedesis (10), suggests another possible role of mCD99: in addition to the simple conveyor role of carrying mCD99L2 to the plasma membrane, mCD99 may also act as a partner to mCD99L2 function at the plasma membrane of leukocyte during transendothelial migration. More data regarding the functions of mCD99 and mCD99L2 need to be accumulated to understand the mechanisms by which they perform their various functions and to address the possibilities raised by this study.

In summary, we demonstrated that a physical interaction between mCD99 and mCD99L2 that influences the molecular trafficking of mCD99. The findings from this study may be of direct relevance to human CD99. Together with previous demonstrations of the roles of mCD99 and mCD99L2 during leukocyte diapedesis, our results provide useful insights into the possible mechanism(s) of controlling transendothelial migration of leukocytes.

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References

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